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STUDIES ON THE MECHANISM OF ACTION OF THE IN VITRO PGRX EFFECT.--ETC(U)
SEP 81 H W SHMUKLER, M G ZAWRYT, E SOFFER

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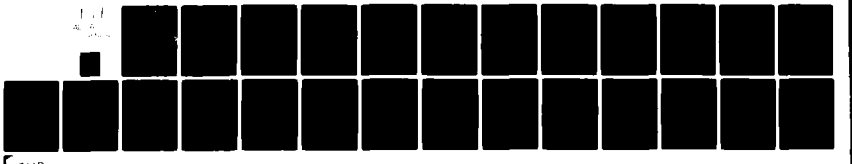

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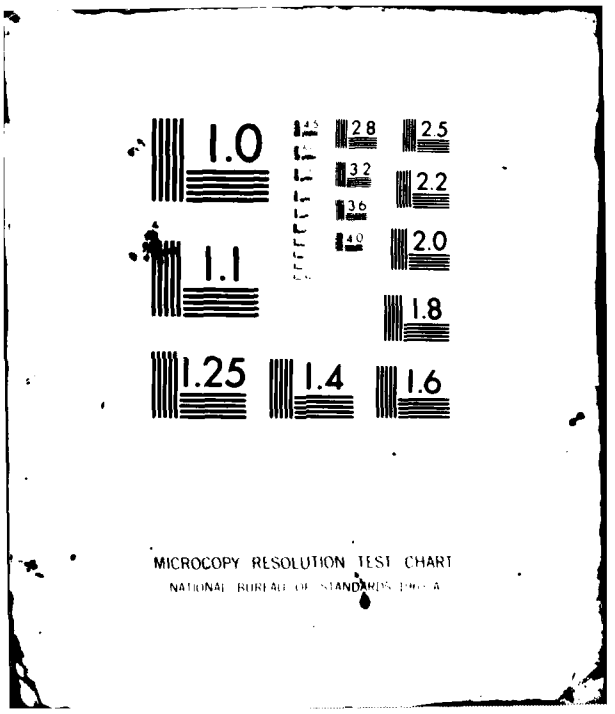
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REPORT NO. NADC-81223-60

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STUDIES ON THE MECHANISM OF ACTION OF THE IN VITRO PGB_x EFFECT

I. COMPOSITION OF REACTION MEDIUM FOR PGB_x EFFECT

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4 September 1981

Phase Report
Airtask No. F58527803
Work Unit No. EH810

APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED

Prepared for
Office of Naval Research
Department of the Navy
Arlington, Virginia 2217

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER NADC-81223-60	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Studies on the Mechanism of Action of the in vitro PGB_x Effect I. Composition of Reaction Medium of PGB_x Effect		5. TYPE OF REPORT & PERIOD COVERED Phase Report 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Herman W. Shmukler, M. G. Zawryt and E. Soffer		8. CONTRACT OR GRANT NUMBER(s) N00014-81-WR10070
9. PERFORMING ORGANIZATION NAME AND ADDRESS Naval Air Development Center Aircraft and Crew Systems Technology Directorate Warminster, PA 18974		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS Airtask: F58527803 Work Unit No.: EH810
11. CONTROLLING OFFICE NAME AND ADDRESS Office of Naval Research Department of the Navy Arlington, VA 22217		12. REPORT DATE 4 September 1981 13. NUMBER OF PAGES
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) UNCLASSIFIED 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

PGB_x
Rat liver mitochondria
Oxidative phosphorylation

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The mechanism of action of PGB_x in RLM oxidative phosphorylation was studied by determining the necessity of each component of the reaction medium. These components maybe classified as essential, e.g. Pi, Mg^{++}, phosphate acceptor and oxidizeable substrate, or non-essential, e.g. KCL and BSA. Although the phosphate acceptor was an absolute requirement for the PGB_x effect, all adenine nucleotides, singly or in combination, as well as the glucose-ADP-hexokinase system were satisfactory. Of the oxidizeable substrates tested —

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20. (Cont'd) using the experimental conditions described here only two did not give positive PGB_x effects: β-hydroxybutyrate and externally reduced NAD. In addition the PGB_x effect can only be demonstrated with isolated mitochondria exposed to hypotonic conditions at 27°C for a time period that will reduce the phosphorylation level to about 5% of that obtained with undegraded mitochondria.

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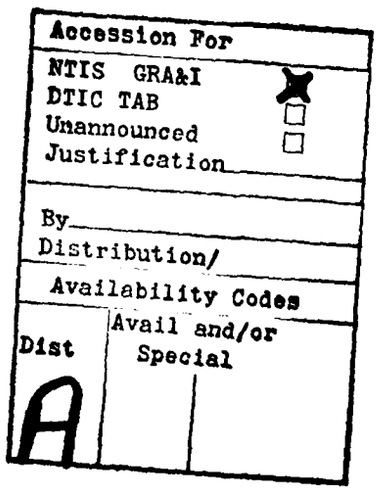

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INTRODUCTION

In 1973, Polis et al (1) first reported the synthesis of PGB_X, an oligomeric derivative of PGB_1. Later they reported that PGB_X conserved oxidative phosphorylation in isolated RLM exposed to degenerative conditions that usually resulted in a loss of phosphorylation efficiency (2, 3). Since in vivo mitochondria exposed to tissue anoxia undergo morphological changes that also result in the loss of phosphorylation efficiency, Polis et al (1) suggested that PGB_X might be effective in the treatment of pathological conditions arising from tissue anoxia. Support for this hypothesis was obtained by (a) Polis and Angelakos (4, 5, 6) who showed that PGB_X treated monkeys survived experimentally induced cardiogenic shock, (b) Polis and Kolata (7, 8) who showed that PGB_X treated rabbits survived experimentally induced cerebral ischemia, (c) Yamazaki et al (9) who showed a beneficial effect of PGB_X on the contraction of ischemic myocardium in dogs, and (d) Moss et al (10) who showed that dogs treated with PGB_X survived lethal cerebral hypoxia.

On the basis of these animal studies it appears that PGB_X may serve as a possible therapeutic agent in the treatment of human diseases in which mitochondrial damage occurs, e.g. myocardial and cerebral ischemia. However before human testing may be undertaken certain basic information concerning the chemical structure and mechanism of action of PGB_X must be delineated. In this series of reports, studies on the mechanism of action of PGB_X at the in vitro mitochondrial level are described. In the first paper of this series the chemical composition of the system for the demonstration of the in vitro PGB_X effect as well as the absolute requirements of the individual components are described.

Note: Abbreviations used in this report are: RLM, rat liver mitochondria; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; Pi, inorganic phosphate; ~Pi, high energy phosphate; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; NADH, reduced nicotinamide adenine dinucleotide

EXPERIMENTAL

Materials: PGB_X was synthesized and purified according to Polis et al (2, 3) and stored as the sodium salt at -20°. For use, Na-PGB_X was dissolved in water, usually at a concentration of 1.0mg/ml, and stored at 3° until used. RLM were isolated from males (supplied by Jackson Laboratories, Bar Harbour, Maine) by a modification of the Hogeboom et al method (11) as reported previously (3). Hexokinase (Type F-300) and adenine nucleotides were supplied by Sigma Chemical Co. (St. Louis, MO).

Analytical: The in vitro PGB_X effect was measured as described previously (2, 3). HPLC of nucleotides was carried out by the method of Shmukler (12). When ATP-glucose-hexokinase system was used the concentrations were ATP, 0.455×10^{-3}M; glucose, 5.68×10^{-3}M; and hexokinase, 6.8 units/ml.

RESULTS

The PGB_X assay system described by Polis et al (2, 3) consists of a preliminary exposure of aged RLM to a hypotonic medium consisting of phosphate buffer, α-ketoglutarate, and $MgSO_4$ at $_pH$ 7.35, with a total osmolarity equivalent to 0.059. After a sufficient period of exposure, which is dependent upon the age and condition of the isolated RLM, the other reactants of the system, namely, AMP, ADP, KCl and BSA were added and phosphorylation measured at the end of 20 minutes. In an effort to elucidate the mode of PGB_X action, the above assay system was explored in detail varying the concentration and composition of reactants in order to define the absolute requirements for demonstrating the PGB_X effect.

The Effect of Omitting Hypotonic Exposure of RLM in the PGB_X Assay.

It is well known that RLM exposed to hypotonic media undergo morphological changes resulting in mitochondrial swelling. As a result of this hypotonic exposure, RLM lose their ability to carry out oxidative phosphorylation. However if this hypotonic medium contains nucleotide acceptor, inorganic phosphate and oxidizeable substrates, as found in State III (13), mitochondria do not swell and are capable of a high degree of phosphorylation. As pointed out by Polis et al (2, 3) the PGB_X effect, is only observed in RLM degraded by exposure to hypotonic media not equivalent to Stage III. Figure 1 shows the phosphorylation ability, in terms of Pi esterfied as a function of added PGB_X to the test system, in which mitochondria were (a) first exposed to hypotonic media for 13 minutes (curve ——, equivalent to normal PGBx assay system) or (b) assayed under State III condition (curve ----).

The normal PGB_x assay as a function of PGB_x exhibits the usual biphasic response curve previously reported by Shmukler et al (14) and Devlin et al (15) in which at low PGB_x concentrations (up to 10µg/reaction) the phosphorylation ability increases with increasing PGB_x concentrations and reaches a maximum between 8 and 12µg. Beyond this range the phosphorylation ability is depressed, and depending upon the age and preparation of RLM may even revert to that level exhibited by RLM in the absence of PGB_x. In the experiments shown in Figure 1 exposure of mitochondria to hypotonic conditions resulted in a drop of esterfied Pi from 6.53 to 0.35µmoles per reaction. In the presence of PGB_x these mitochondria were able to show an increase Pi esterification with increased amounts of PGB_x (0 —— 8µg/reaction); between 8 and 40µg PGB_x the response was maximal and at the highest concentration tested, 80µg, the stabilization of phosphorylation was 2.2µmoles or 33% of non-exposed RLM. In contrast to this, the addition of increasing amounts of PGB_x, to the mitochondrial assay system in which preliminary degradation was omitted, resulted only in a slight inhibition of phosphorylation. Even at the highest PGB_x concentration used, the inhibition of phosphorylation amounted only to about 25%. These results confirm the findings of Polis et al (2, 3) that hypotonic exposure of RLM is essential to demonstrate the in vitro PGB_x effect.

The Component Requirements of the in vitro PGB_x Assay System.

It is obvious that certain constituents must be present in order to demonstrate oxidative phosphorylation with isolated RLM. These essential constituents are inorganic phosphate, phosphate acceptor and oxidizeable substrate. All other components are usually added to optimize the reaction. Since the PGB_x effect consists of 2 steps (a) degradation of RLM and (b) oxidative phosphorylation, it was of interest to determine the effect of the non-essential constituents on the PGB_x effect as well as defining the specific requirements of the essential constituents.

Bovine serum albumin requirement: In 1953 Shmukler and Polis (16) first reported the stimulation of oxidative phosphorylation by the addition of BSA to aged RLM stored at 3°. This BSA stimulation was later attributed to the non-specific binding of long chain fatty acids, known uncouplers of oxidative phosphorylation, which were released during storage of RLM. In the PGB_x assay, aged degraded RLM are used and Polis et al (2, 3) postulated that BSA should eliminate intereference due to fatty acid release. The effect of BSA on the PGB_x effect is shown in Figure 2, in which the PGB_x effect was assayed over a wide range of PGB_x concentrations in the presence and absence of BSA in Step 2 of the assay system. The omission of BSA from the standard test system does not alter the usual biphasic PGB_x response, but rather it does affect the degree of phosphorylation, in that the addition of BSA results in a higher degree of phosphorylation at all levels of PGB_x tested. These findings suggest that BSA is not essential to demonstrate the PGB_x effect but rather does provide optimal phosphorylation response.

Inorganic salt requirements: The inorganic constituents of the PGB_x assay system are phosphate buffer, $MgSO_4$ and KCl. Since inorganic phosphate is an obvious absolute requirement in the measurement of RLM oxidative phosphorylation, only $MgSO_4$ and KCl were investigated in this study. Their absolute requirements were determined by carrying out the in vitro PGB_x assay in which the PGB_x concentration was varied over those values known to yield the biphasic response, and in which either inorganic constituent was omitted. Figure 3 shows the effect of omitting KCl (curve ■ — ■) and omitting $MgSO_4$ (curve • — •) as compared to the standard assay system (curve + — +). When KCl was omitted the Pi esterified as a function of PGB_x concentration was similar to that usually obtained with the standard system, except that the response was slightly decreased. In contrast when $MgSO_4$ was omitted no phosphorylation was detected.

Nucleotide requirement: Polis et al (2, 3) used an equal molar mixture of AMP and ADP as a ∿Pi acceptor system to demonstrate the in vitro PGB_x effect. In order to determine if this nucleotide mixture was unique to the PGB_x effect, assays were carried out using the adenine nucleotides individually. In addition the PGB_x effect was evaluated with the ADP-glucose-hexokinase system, since this system is preferred by most investigators in the field of oxidative phosphorylation. Figure 4 shows the PGB_x effect as a function of PGB_x concentration when AMP alone (• — •), ADP alone (■ — ■), ATP alone (× — ×), ADP-glucose-hexokinase (◆— ◆ and the standard AMP-ADP system (+ — +). A comparison of the results for AMP alone, ADP alone and the standard system show an almost equivalent PGB_x effect. When ADP-glucose-hexokinase system was used a slight increase in phosphorylation was observed (ca 12%), however the PGB_x response curve appeared similar in shape to the standard curve. In the case of ATP only as acceptor, the esterified phosphate calculated gave negative values except between 2 and 9μg PGB_x/ml. This may be explained on the basis that ATPase which is normally latent in fresh RLM, becomes active in aged or degraded RLM to hydrolyse the added ATP to ADP and Pi. The de novo ADP is then available as acceptor for ∿Pi while the free Pi contributes to the total Pi pool. This Pi pool then represents the equilibrium between the phosphorylation-dephosphorylation processes taking place. Since the Pi esterified is calculated by subtracting the analysed Pi from the added Pi, then when ATP was used as acceptor, the Pi pool was much larger than the added Pi and in using this latter value to calculate Pi esterification, negative values would result. An examination of this curve shows that the shape of the PGB_x effect vs PGB_x concentration is similar to the standard curve, except that the curve originates below zero. An estimate of the total Pi pool can be made using the Pi concentration found in the reaction carried out in the absence of PGB_x. When this value was substituted for the added Pi, the shape and intensity of the PGB_x effect curve was almost identical to the curve for the standard assay system. To show that this

interpretation was valid, the adenine nucleotide composition of each of the PGB_X assay mixtures was analysed by HPLC (see "Methods"). The results of these analyses are plotted in Figure 5. In this figure the arrow pointing to the ordinate indicates the ATP concentration before the addition of RLM. After RLM are added to the test system without PGB_X, there is an immediate drop in the ATP concentration consistent with the concept of ATPase action. With increasing concentrations of PGB_X the concentration of ATP increases, levels off and then decreases. The resulting curve is similar to that obtained when the Pi esterified is measured using the standard system.

In previous report Lehninger et al (18) demonstrated phosphorylation associated with the oxidation of external NADH by RLM. For this phosphorylation to take place it was necessary to add cytochrome C or to subject the RLM to some structural damage. The similarity in the structural state of the RLM used in the above study and that used by Polis et al (2, 3) for the PGB_X effect, suggested that this system also would be of value to demonstrate the PGB_X effect. However when PGB_X was assayed in the system described by Lehninger et al (18) no phosphorylation was found with aged RLM. In a more recent study Maley (19) demonstrated that using alcohol dehydrogenase to generate NADH continuously, phosphorylation associated with the oxidation of NADH could be easily demonstrated. This system was modified slightly in this laboratory in order to measure the effect of PGB_X on phosphorylation associated with oxidation of NADH by RLM. Each test reaction system contained 6μmoles ATP, 40μmoles Pi buffer $_p$H 7.4, 15μmoles $MgSO_4$, 10μmoles DPN, 85μmoles of ethanol, 30μmoles KF in a total volume of 2.53 ml. This reaction mixture was equilibrated at 27° and then 7.5mg RLM were added. When PGB_X was tested, it was added to the test mixture prior to the addition of RLM. The effect of PGB_X was tested under conditions where RLM were not degraded, and also after 8' degradation. To initiate the reaction a mixture containing 50μmoles glucose, 15 units of hexokinase and 15 units of alcohol dehydrogenase were added and the reaction allowed to proceed for 20 minutes. At the end of this time the Pi remaining was determined by withdrawing 0.2 ml from each test system which was then added to 1.0 ml 5% $HClO_4$. The solution was mixed and centrifuged. The Pi content of the protein-free filtrate was measured by the Fiske and Subbarow method (20). The esterified Pi was then calculated from the difference between the added Pi and the remaining Pi. Figure 6 shows a plot of the esterified phosphate found in the test mixtures containing various amounts of PGB_X. As seen in this figure there was no difference between the Pi esterified found in the presence of absence of PGB_X, or using intact or degraded RLM. The implication of these results is that since phosphorylation associated with the oxidation of external NADH is not modified by the addition of PGB_X, then there is no in vitro PGB_X effect with this system.

Substrate requirements: In the original assay system for the demonstration of the PGB_X effect Polis et al (2, 3) used α-ketoglutarate as oxidizeable substrate. Since no information was available concerning the use of other substrates, it was of interest to determine the universality of the PGB_X effect with other substrates generally used in mitochondrial oxidative phosphorylation. For this purpose the PGB_X effect was determined by substituting various substrates for α-ketoglutarate in the assay system. The concentration of the test substrates was identical to that of α-ketoglutarate in the normal assay. Figure 7 shows the Pi esterified for a number of substrates as a function of time of treatment of RLM to the conditions of step 1 of the PGB_X assay system. With the RLM preparation used in this experiment, the time required to reduce the phosphorylation level to that required for the PGB_X assay, was 15 minutes for α-ketoglutarate, pyruvate and malate and 7 minutes for fumarate. In contrast when isocitrate was the substrate the phosphorylation level was reduced only by 1/3 even after 30 minutes exposure. Figure 8 shows the PGB_X concentration curves obtained using the substrates from figure 7, at the comparable degradation times for each substrate listed above. As seen in this figure the biphasic PGB_X concentration response curve appeared similar for all substrates other than isocitrate tested, which yielded a PGB_X response curve that did not drop at high concentrations. When pyruvate was used as substrate the PGB_X response curve showed the typical biphasic response, except that the phosphorylation level at the maximum was higher than with any other substrate tested and in addition the drop in phosphorylation appeared to be sharper. Figure 9 shows PGB_X response curves when succinate, glutamate, and β-hydroxybutyrate were the substrates as compared to α-ketoglutarate. With the RLM used in this figure the required degradation time was 11 minutes. Under these conditions succinate and glutamate gave a lower PGB_X response curve than with α-ketoglutarate. In contrast β-hydroxybutyrate showed no PGB_X effect at any PGB_X concentration tested.

DISCUSSION

The purpose of this study was to compile in one report the experimental conditions necessary to demonstrate the PGB_X effect on RLM oxidative phosphorylation. In addition it was hoped that such a summation would point out avenues of research that might lead to the elucidation of the mechanism of the PGB_X effect.

In the original report of the PGB_X effect Polis et al (2, 3) used a preliminary pretreatment of RLM at 27° (step 1) in order to alter the mitochondrial structure so that the phosphorylation ability was markedly reduced; when these RLM were pretreated in the presence of PGB_X their ability to carry out oxidative phosphorylation was maintained approximately at the same level as untreated RLM.

The addition of PGB_X to test systems in which RLM were not pre-exposed to hypotonic conditions was reported by Polis et al (3), and confirmed here (Figure 1), to have only a minimal effect on the degree of phosphorylation. Even though PGB_X is a complex long chain fatty acid and the salts exhibit detergent properties (16, 17) it appears to have no effect on the oxidative phosphorylation of intact RLM in contrast to ordinary long chain fatty acids that are known to uncouple oxidative phosphorylation.

The components of the test system for the PGB_X effect have been investigated in detail and may be classified as essential components, i.e., absolute requirement for demonstration of oxidative phosphorylation and/or the PGB_X effect, or unessential components, i.e., required for maximal PGB_X effect only. In the essential class obviously Pi, Pi acceptor and oxidizeable substrate are required. In addition Mg^{++} appears to be an essential component since in its absence, no phosphorylation was observed. This agrees with the accepted concept that the Mg^{++} nucleotide complex is the form in which nucleotides are active in enzyme systems. Consequently in the absence of Mg^{++} the nucleotide can not function as an acceptor for Pi during oxidation of substrate and thus phosphorylation can not be coupled to oxidation. As shown in this study any of the adenine nucleotides as well as ADP-glucose-hexokinase may serve as acceptor for $\sim$Pi generated during oxidation of substrate. Similarly oxidative phosphorylation requires an oxidizeable substrate to supply the energy for the generation of $\sim$Pi. The substrates tested in this study all appear effective in maintaining RLM oxidative phosphorylation, however they do show a variable response to the action of PGB_X. Notably β-hydroxybutyrate cannot support oxidative phosphorylation of pretreated RLM and consequently shows no PGB_X effect. However more recent studies have shown that β-hydroxybutyrate may be used to assay PGB_X but only with RLM exposed to very short degradation names, e.g., 2 minutes. In contrast, isocitrate does show a PGB_X effect and in addition appears to support oxidative phosphorylation of RLM pretreated for an extended period, a pretreatment that results in uncoupling of oxidative phosphorylation using the usual metabolites e.g., α-ketoglutarate, pyruvate, malate, fumarate and glutamate. Finally pyruvate appears to yield the maximal PGB_X effect in terms of activation, however at high concentrations of PGB_X, the phosphorylation level drops at much lower PGB_X levels. It is interesting to note that only two of the substrates tested were found not to support the PGB_X effect but for different reasons. In the case of β-hydroxybutyrate, hypotonic pretreatment of RLM resulted in a complete uncoupling of oxidative phosphorylation to a point as which PGB_X had no protective effect on the RLM. On the other hand when externally reduced NAD was the substrate, the degree of uncoupling attained with the pretreatment of RLM was insufficient to demonstrate the PGB_X effect.

The compontents of the test system classified as non-essential are BSA and KCl. The addition of BSA to the test system increases the degree of phosphorylation without affecting the shape of the PGB_x response curve. The explanation for this phenomenon is probably that BSA binds fatty acids that are freed during storage of RLM at 0^o in sucrose of during the pretreatment with hypotonic medium. The neutralization of these fatty acids, i.e., uncouplers of oxidative phosphorylation, permits the demonstration of higher levels of phosphorylation. The addition of KCl to the reaction mixture appears to only offer a slight increase in the degree of phosphorylation.

The results of this study show that the PGB_x effect can only be shown with RLM pretreated with a hypotonic medium containing Pi, Mg^{++} and a suitable oxidizeable substrate and that this pretreatment must be of such duration as to inhibit the control reaction only to the point that phosphorylation is decreased to about 5% of the intact RLM. If the pretreatment is allowed to proceed beyond this time period added PGB_x may not be effective. In addition to this pretreatment it is essential that all the components for the demonstration of oxidative phosphorylation are present.

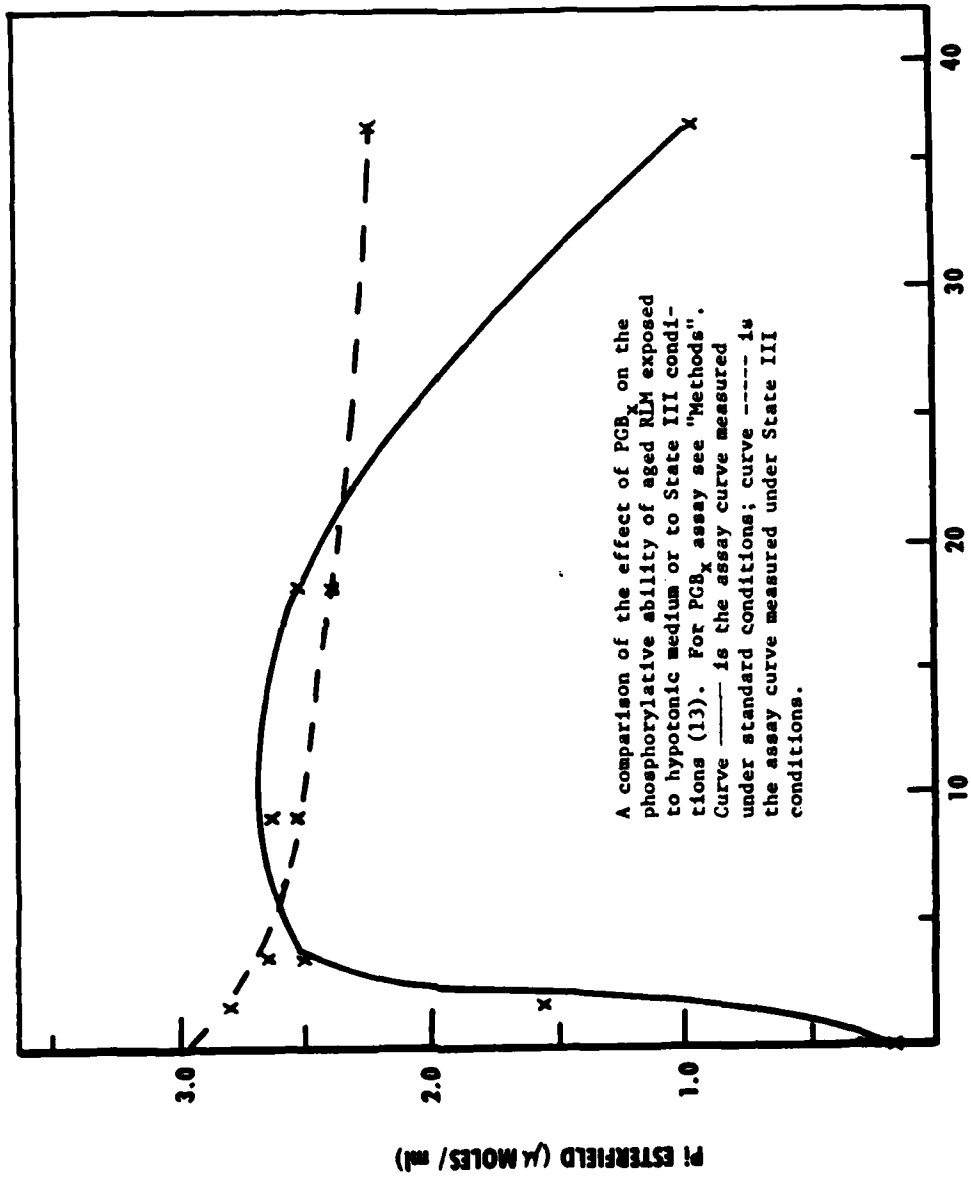

Figure 1 - A Comparison of the Effect of PGB_X on the Phosphorylative Ability of RLM Exposed to Hypotonic Medium or State III Conditions

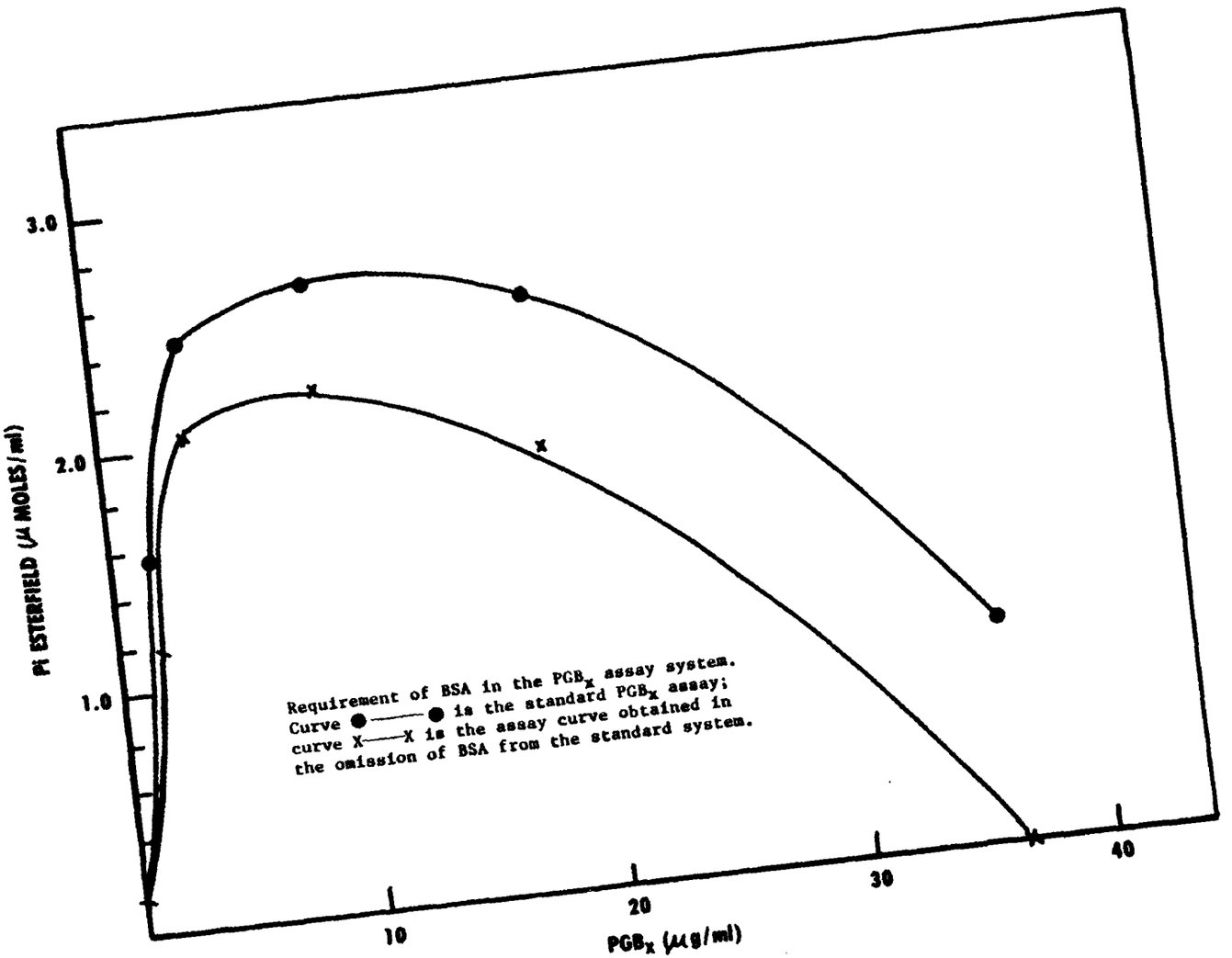

Figure 2 - Requirement of BSA in the PGB_x Assay System

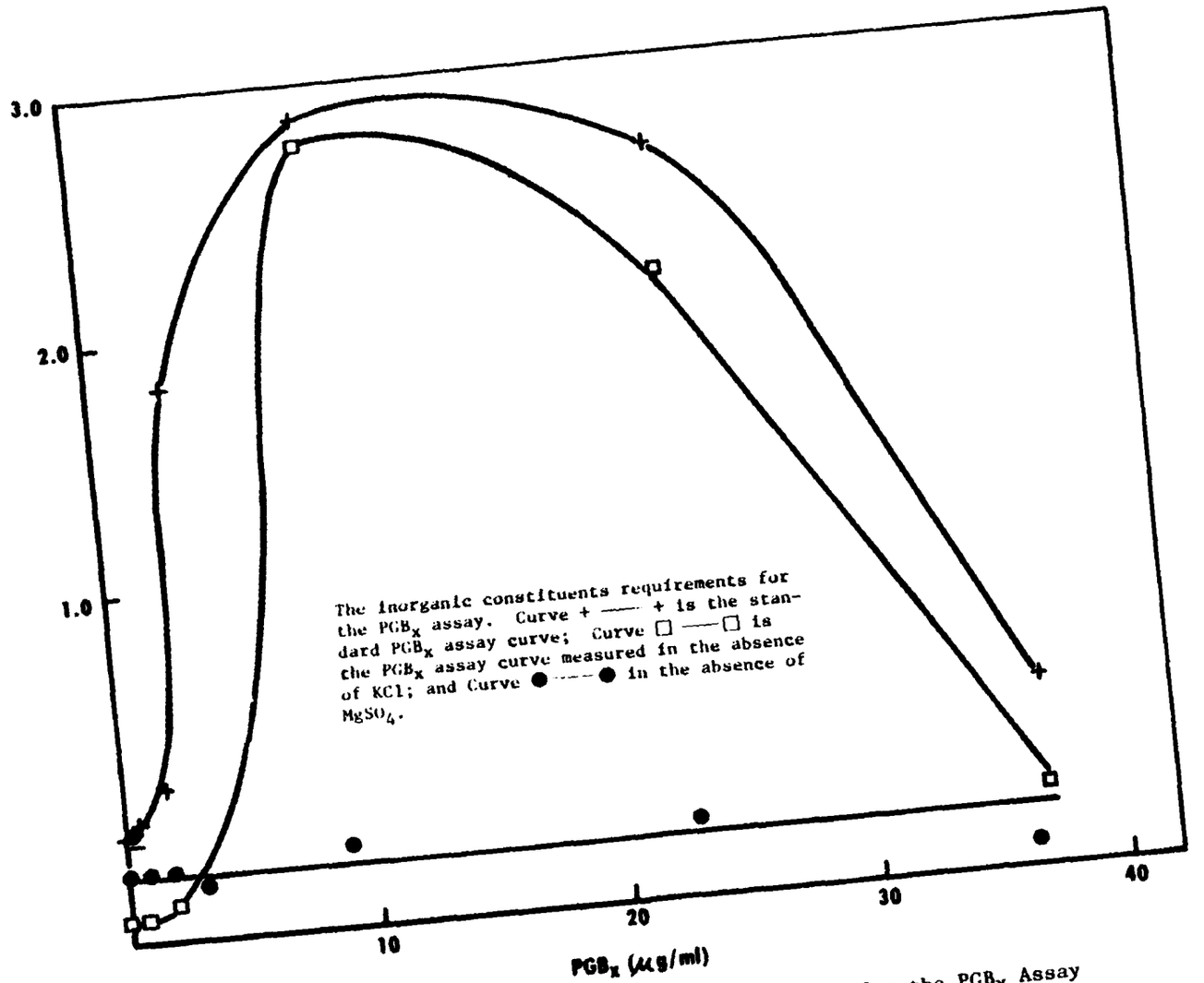

Figure 3 - The Inorganic Constituents Requirements for the PGB$_x$ Assay

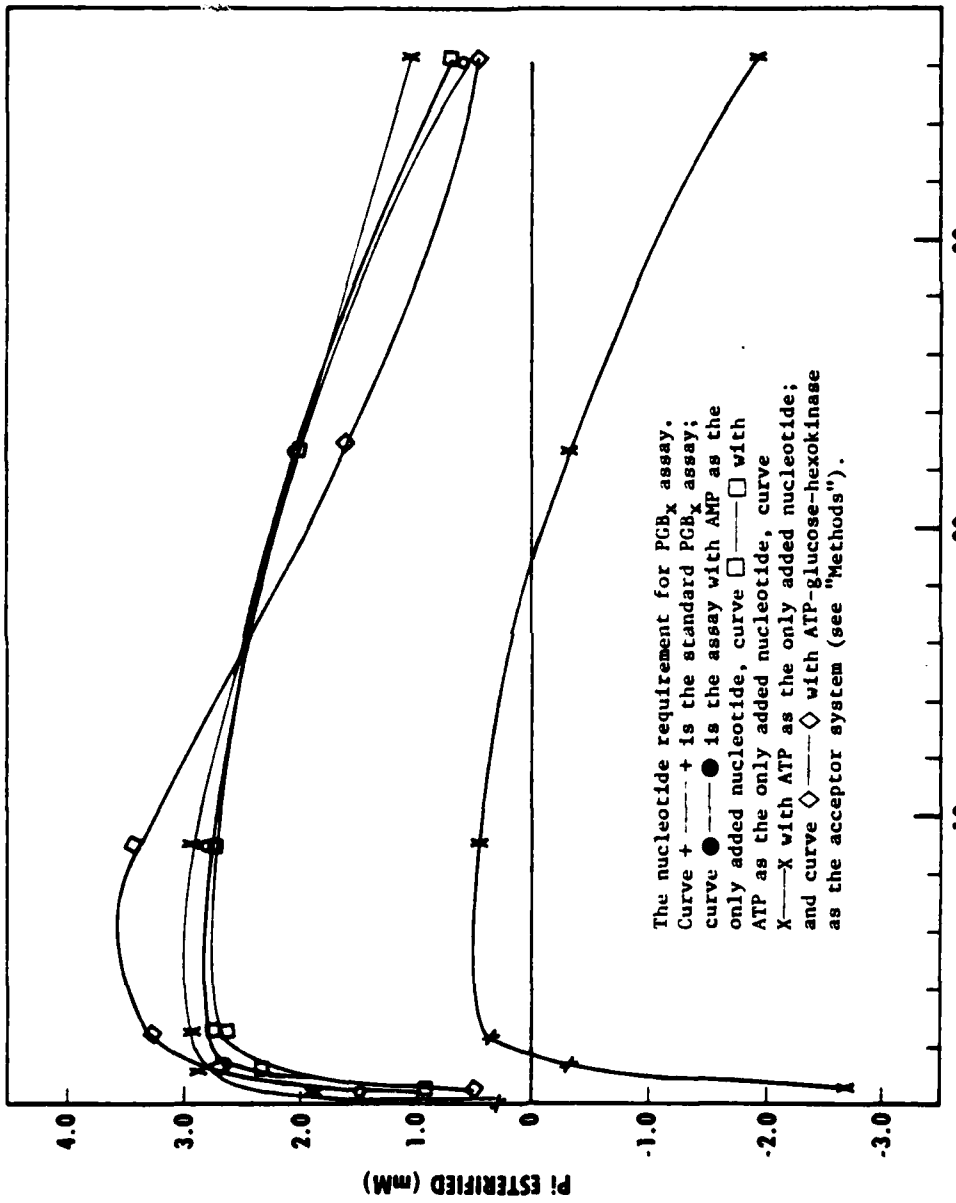

Figure 4 - The Nucleotide Requirement for the PGB_x Assay

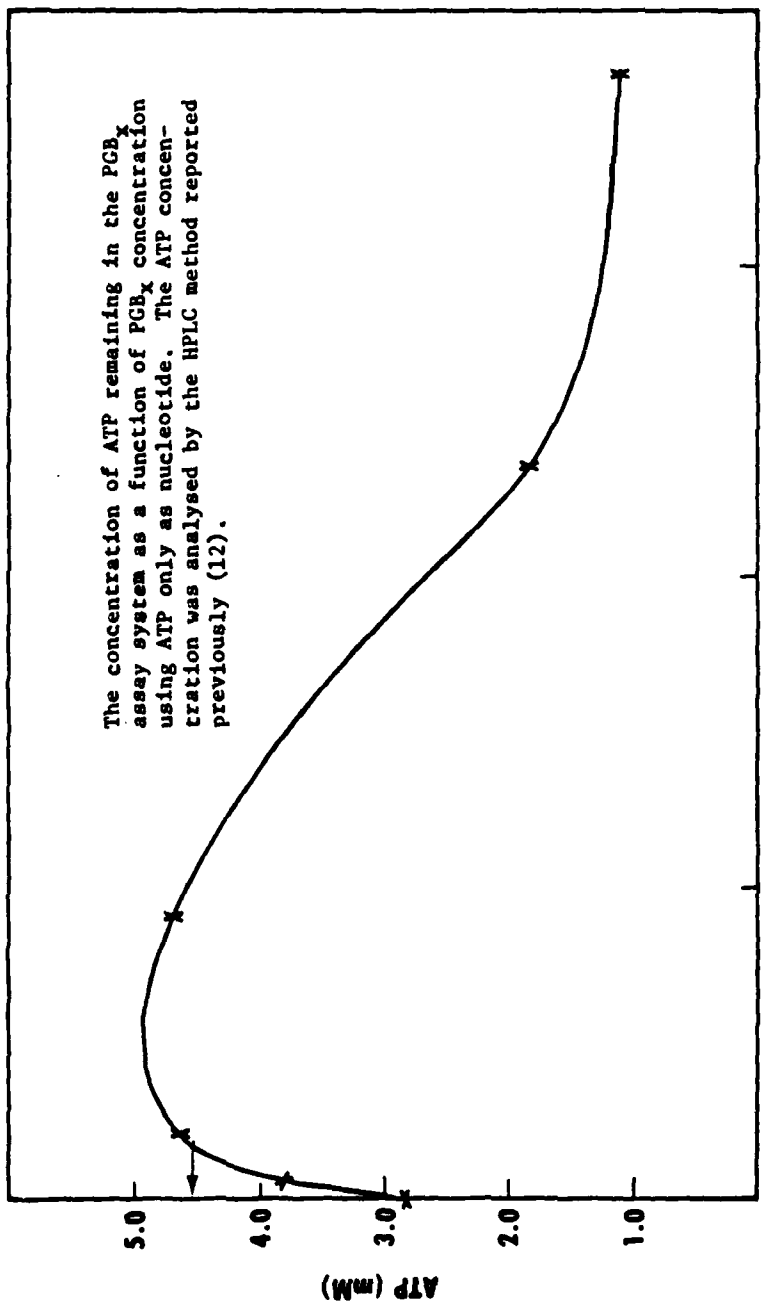

Figure 5 - The Concentration of ATP Remaining in the PGB_x Assay System as a Function of PGB_x Concentration

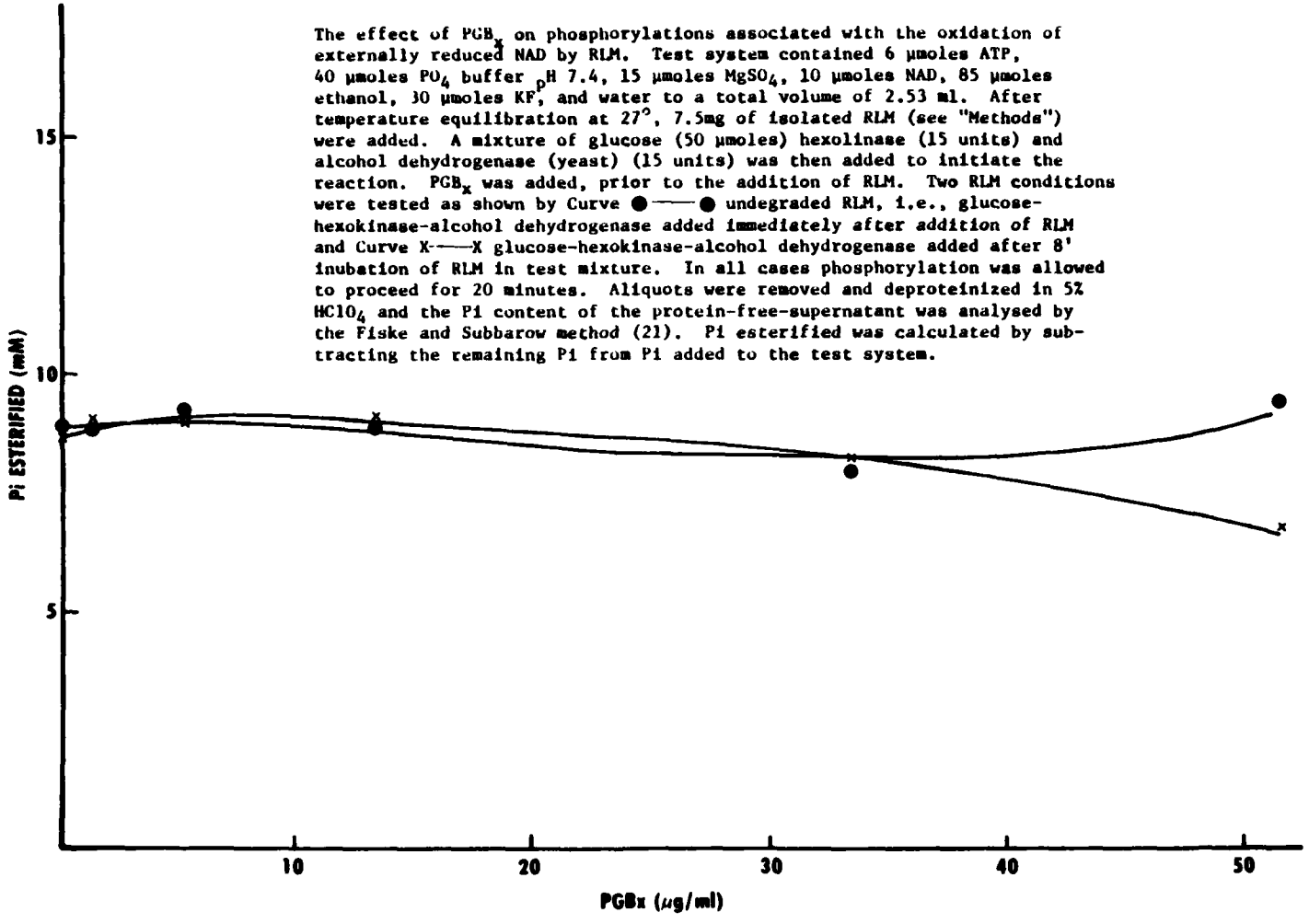

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Figure 6 - The Effect of PGB_x on Phosphorylations Associated with the Oxidation of Externally Reduced NAD by RLM

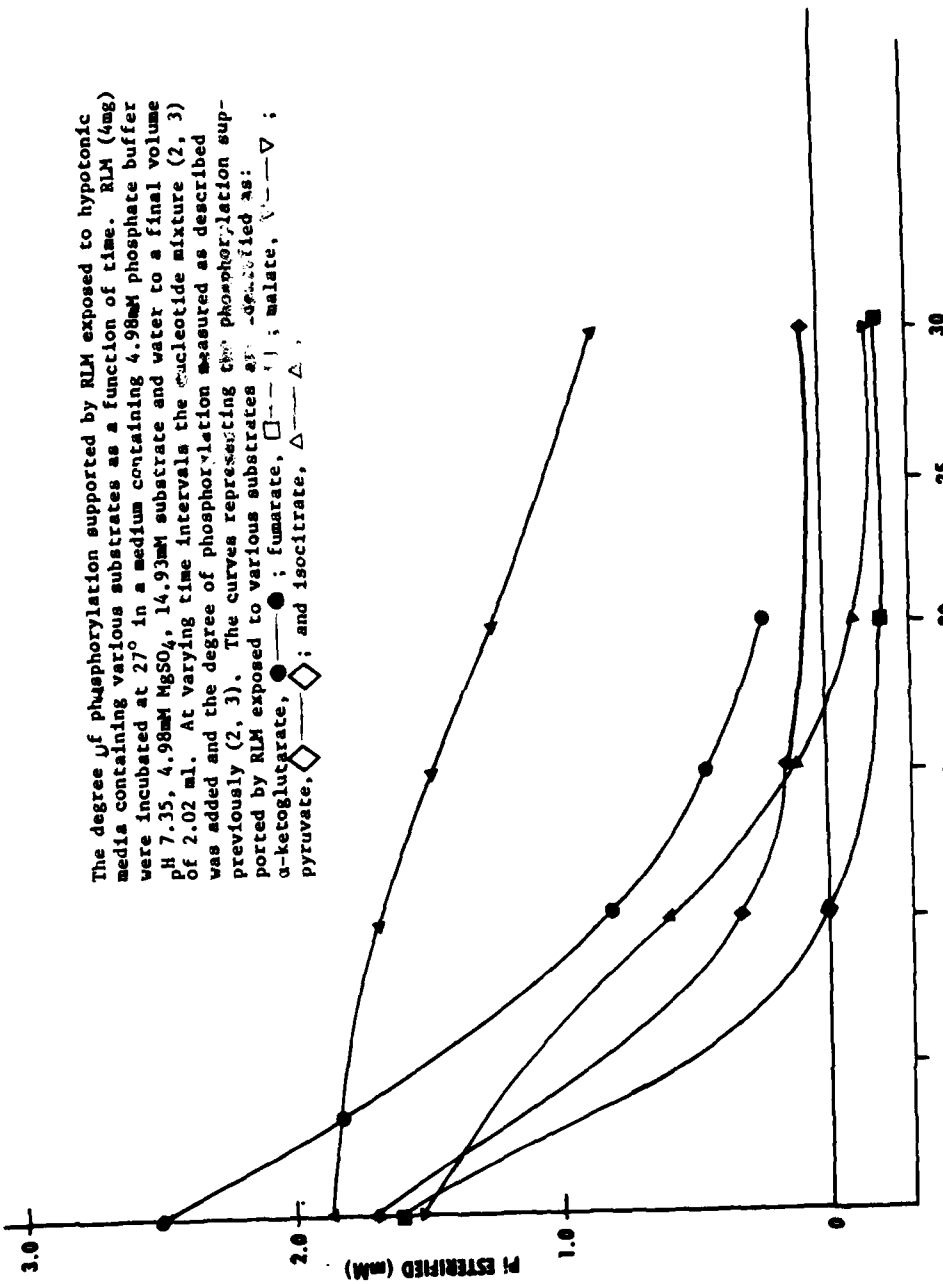

Figure 7 - The Degree of Phosphorylation Supported by RLM Exposed to Hypotonic Media as a Function of Time

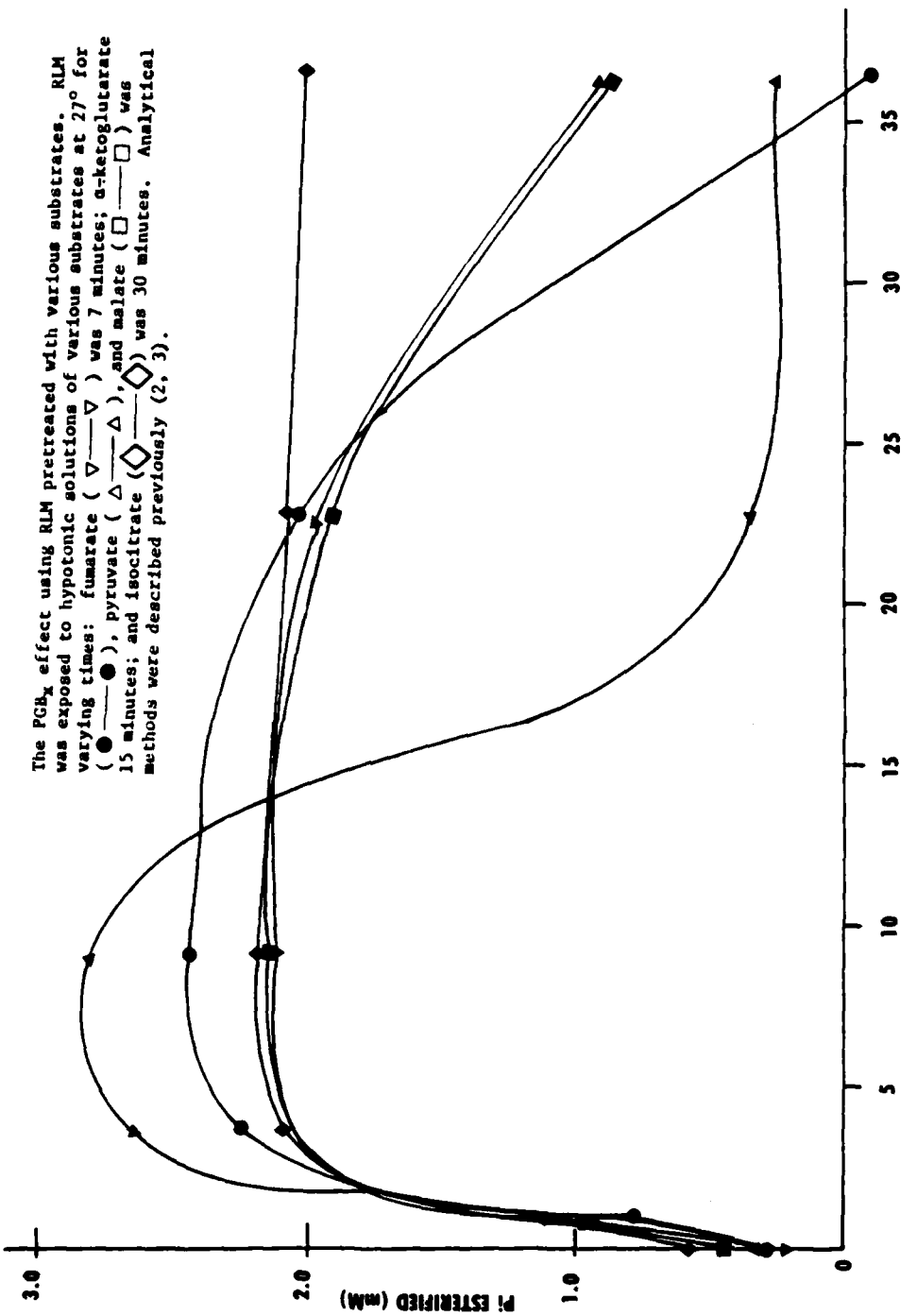

Figure 8 - The PGB_x Effect Using RLM Pretreated with Various Substrates

The PGB_x effect using RLM pretreated with various substrates. RLM was exposed to hypotonic solutions of α-kitoglutarate (●——●), β-hydroxybutyrate (◇——◇), glutamate (□——□), and succinate (△——△) for 11 minutes. The PGB_x effect was then measured as described (2, 3).

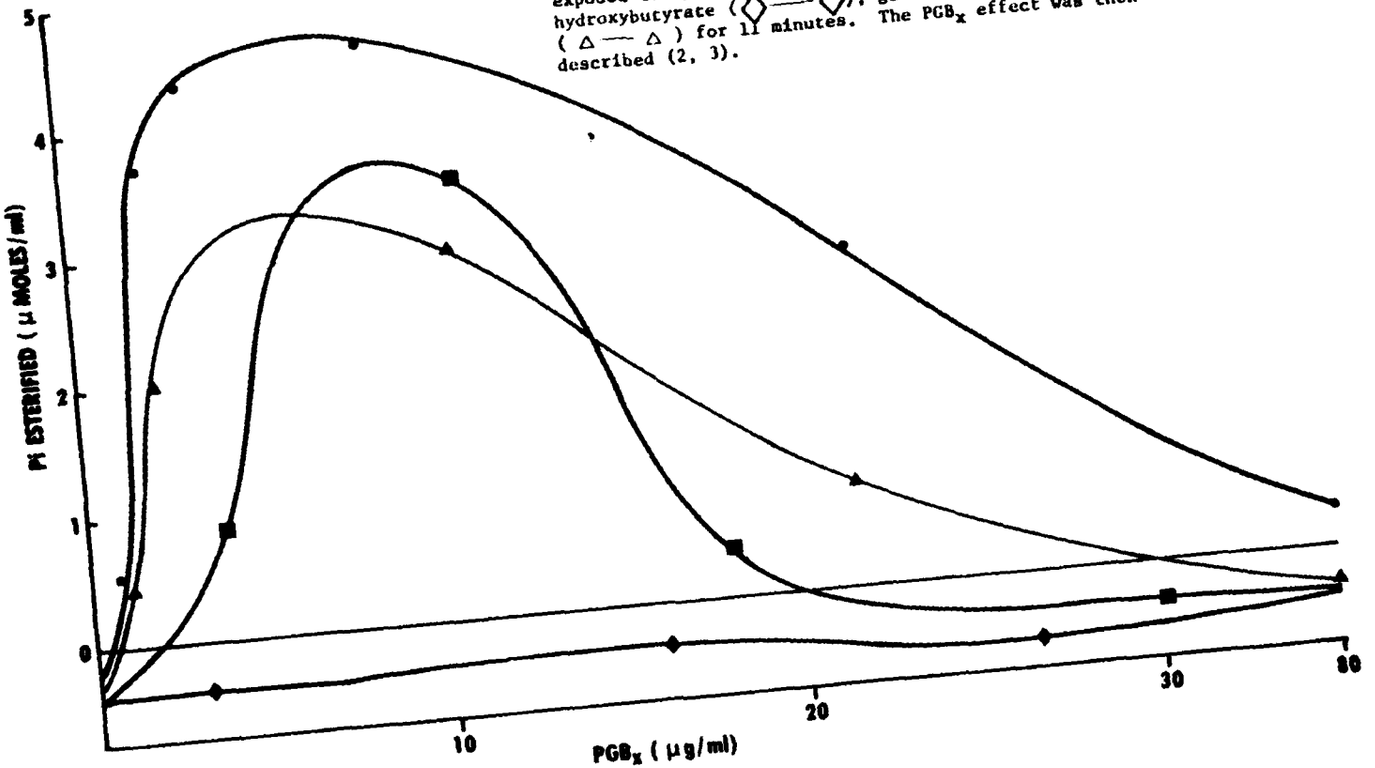

Figure 9 - The PGB_x Effect Using RLM Pretreated with Various Substrates

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